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The Construction and Analysis of CeRNA Network and Patterns of Immune Infiltration in Acute Myeloid Leukemia

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Abstract

Objectives: Acute Myeloid Leukemia (LAML) is a rapidly progressive malignant blood disorder that is prone to relapse and has a relatively low survival rate after treatment. There is an urgent need to find new biomarkers that may improve the survival prognosis of patients. In recent years, immunotherapy has become a hot topic in cancer treatment, but its application in LAML is still in the exploratory stage.

Methods: By screening differentially expressed mRNAs and associated miRNAs and circRNAs based on TCGA, GTEx and GEO databases, constructing competitive endogenous RNA (ceRNA) networks using Metascape , string, starBase, GSEA; using Kaplan-Meier survival analysis curves and Cox proportional risk model and column to predict prognostic value; cibersort algorithm was used to map gene and immune cell correlations.

Results: We screened 6 mRNAs, 4 miRNAs and 11 circRNAs based on TCGA, GTEx and GEO, and constructed a ceRNA network based on their interactions. The CIBERSORT algorithm shown 22 immune cells were significantly associated with LAML pathogenesis, and multiple R packages were used to find that immune cells and immune checkpoints were significantly associated with key genes of the ceRNA network, and key genes of the ceRNA network were significantly associated with each subtype of LAML.

Conclusions: In this study, we hypothesized that LAML

pathogenesis may be associated with the interaction of 6 mRNAs and 4 miRNAs. These 6 mRNAs may be novel potential biomarkers of LAML.

Keywords: LAML; Tissue-Specific Expressed Genes; Cerna Network; Biomarkers; Immune Cell

Introduction

Acute Myeloid Leukemia (LAML) is a malignant blood disorder that usually results from the abnormal proliferation of naïve granulocytes or primitive hematopoietic progenitor cells in the bone marrow [1]. Symptoms of LAML include malaise, fever, infection, anemia, bone pain, subcutaneous bleeding, diarrhea, vomiting, loss of appetite and myelodysplasia resulting in enlargement of the spleen and liver [2,3]. LAML is a rapidly developing leukemia that can be life-threatening if left untreated [4]. Traditional treatments include chemotherapy, radiation therapy and bone marrow transplantation, but these methods do not completely cure all patients [5]. In recent years, immunotherapy has made some progress in the treatment of LAML as a new therapeutic approach [6]. Although some initial results have been achieved with immunotherapy in the treatment of LAML, there are still many challenges and problems. For example, CAR-T cell therapy is costly and some serious adverse effects, such as cytokine release syndrome, may occur during the treatment [7,8] . In addition, the indications and treatment protocols for immunotherapy in the treatment of LAML still need further research and improvement, and there is also a need to strengthen the early diagnosis and prevention of LAML to improve the cure rate and survival quality of patients.

In recent years, an increasing number of studies have shown that competitive endogenous RNA (ceRNA) networks play an important role in cancer and have become one of the hot spots in cancer research [9]. CeRNA is a molecular mechanism by which non-coding RNAs, long-stranded non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) regulate gene expression by competing for shared microRNAs (miRNAs) [10]. First, the study of ceRNA networks in cancer has revealed many new cancer targets and biomarkers [11]. It has been found that many tumor-associated lncRNAs and circRNAs are able to interact with miRNAs as ceRNA components, thus affecting the basic biological processes such as growth, proliferation and apoptosis of cancer cells, and thus these ceRNAs have an important role in the process of cancer development and progression [12]. It has been found that in hepatocellular carcinoma, lncRNA *MALAT1* affects the growth and invasion of hepatocellular carcinoma cells by regulating the expression of *miR-195* and *miR-26a* [13]; in gastric carcinoma, the regulatory network of lncRNA *H19* and *miR-378-5p* is involved in the development and progression of gastric carcinoma [14]. Second, the study of ceRNA networks in cancer also contributes to the understanding of the mechanisms of cancer onset and progression [15]. Besides, ceRNA networks can also provide new ideas for cancer therapy. Through the study of ceRNA networks, new cancer targets and therapeutic targets can be discovered, and some genes or signaling pathways associated with cancer drug resistance can also be identified to provide guidance for clinical treatment [16]. It has been found that by targeting miRNAs or lncRNAs in certain ceRNA networks, the proliferation and metastasis of cancer cells can be inhibited, and it has the potential to be a new tumor treatment [17]. In conclusion, ceRNA network has an important role in cancer research, and through the in-depth study of ceRNA network, we can better understand the mechanism of cancer occurrence, discover new targets and biomarkers, and also provide new ideas and methods for cancer treatment, which has a broad application prospect.

More importantly, there have been some advances in the study of ceRNA networks in LAML. lncRNAs such as *VPS9D1-AS1* and *LINC00649* can regulate the expression of their downstream target genes by competitively binding to miRNAs, thereby affecting the proliferation and metastasis of LAML cells [18,19]. These studies provide important clues to gain insight into the mechanisms of LAML occurrence and the search for new therapeutic strategies. However, these studies were based on the ceRNA network constructed by lncRNA. In our study, we explored the possibility that circRNAs may influence the development of LAML by competitively binding to miRNAs to regulate the expression of their downstream target genes, potentially providing novel biomarkers for LAML.

Materials and Methods

LAML expression profile mRNA data were obtained from the TCGA (https://tcga-data.nci.nih.gov/tcga/) database and GTEx (https://www.gtexportal.org/) database, miRNA data

and circRNA data (GSE142699, GSE94591, GSE116617 and GSE163386) were downloaded from the GEO database.

We identified differentially expressed genes (DEG), miRNA (DEmiRNA) and circRNA (DEcircRNA) using the Linear Models for Microarray data (Limma) package61 in R. Adjusted p <0.05 and log2 (fold change) ≥2 or ≤2 were used to identify DEG, and p <0.05 and log2 (fold change) ≥1 or ≤1 to identify DEmiRNA and DEcircRNA. Meanwhile, the volcano maps of DEG, DEmiRNA and DEcircRNA were analyzed with the R gplots program.

The PPI network of DEG was constructed separately using the search tool of string database (https://string-db.org/). Hub genes with high connectivity in PPI networks are identified using Cytoscape software's CytoHubba plugin.

Co-expression analysis of miRNA-mRNA, miRNA-circRNA and mRNA- circRNA pairs identified in this study were evaluated in the LAML cohort of the starBase database. Gene pairs with |r|>0.1 and p<0.05 were used as criteria for further analysis.

Due to the negative correlation between miRNAs and their targets and the positive correlation between mRNAs and lncRNAs/circRNAs [20], we established a circRNA-miRNA-mRNA regulatory network. To deeply investigate the molecular mechanism of LAML pathogenesis, we performed single-gene enrichment analysis of mRNAs in the network using GSEA software. Only the terms with a p < 0.05 and false discovery rate (FDR) q < 0.25 were considered statistically significant.

Then we used the cibersort algorithm to plot box plots, heat maps, and stacking plots for 28 immune cell types. Using KM and Cox analysis to assess the prognostic value of all biomarkers and detect prognostically relevant cell types. The relationships among 22 immune cells and between ceRNA key factors and immune cells were calculated using Pearson correlation coefficients. Using the R heatmap package to generate heatmaps of immune infiltration levels. The ggplot2 package in R software can be used to generate Spearman correlation scatter plots between ceRNA network risk scores and tumor-invasive immune cells in LAML. Correlation plots for all variables are created using the corrplot feature of the corrplot R package.

Results

Identify important DEGs in LAML

In this study, the TCGA and GTEx datasets were collated together for analysis to obtain the DEG of LAML tissue. As shown in the volcano figure, 3096 differential genes were screened in the result display of mRNA DEGs, including 1538 high expressed genes and 1558 low expressed genes (Figure 1A).

In order to analyze the relevant functions of the differential genes in depth, we subjected the differential genes to survival analysis and obtained a total of 61 differential genes with prognosis and 15 genes with significant prognosis (p <0.05), among which *PXN*, *MCOLN1*, *FGR*, *RHOA*, *BID*, *MYL6*, *TNFRSF1B*, *PRKCH*, *NFKB2*, and *FCN1* with high expression were significantly associated with poor prognosis in LAML patients, and low expression of *MAP3K1*, *MPO*, *RGS1*, *HGF*, and R*ANBP9* was associated with poor prognosis in LAML (Figure 1B). To understand the interactions between the identified DEGs, we screened these 15 hub genes according to Cytosxape's cytohubba plugin based on the above differential genes with significant prognosis and found some interactions between these 15 genes (Figure 1C).

Identification and validation of key miRNAs

We analyzed the GSE142699 dataset to obtain the DEmiRNA of the LAML organization. As shown in the volcano plot, a total of 91 differential genes were screened, including 47 up-regulated genes and 44 down-regulated genes (Figure 2A). Next, we analyzed a total of 54 target miRNAs in 15 genes using the Starbase database to construct and visualize miRNA-mRNA interrelationships using Cytoscape. Then, we performed survival analysis on these 54 target miRNAs and showed that 4 miRNAs, *hsa-miR-362-3p*, *hsa-miR-151a-5p*, h*sa-miR-107* and *hsa-miR-19a-3p*, had significant prognostic differences (Figure 2C). The miRNA-mRNA interrelationships of these four miRNA genes with differential prognosis and their corresponding hub genes were finally constructed using Starbase and Cytoscape again, and the network consisted of seven miRNA-mRNA relationships, including four miRNAs and six mRNAs (Figure 2D). Among the six mRNAs, Cox risk regression analysis based on LAML patients identified *MYL6* and *MAP3K1* as prognostic biomarkers,in which *MYL6* had a poor prognosis and *MAP3K1* had a good prognosis (Figure

2E).

Construction of circRNA-miRNA-mRNA network

We first looked for differentially expressed circRNAs in LAML patients in three datasets, GSE94591, GSE116617 and GSE163386, and generated volcano maps based on these genes. As a result, a total of 576 differential genes were screened, including 337 up-regulated genes and 39 down-regulated genes (Figure 3A).

Based on the above four miRNAs with significant prognostic differences, we used the starBase database to predict potential circRNAs upstream of *hsa-miR-362-3p*, *hsa-miR-151a-5p*, *hsa-miR-107* and *hsa-miR-19a-3p*, of which four up-regulated circRNAs may bind to hsa *miR-19a-3p*, three up-regulated circRNAs may bind to *hsa-miR-362-3p*, three down-regulated circRNAs may bind to *hsa-miR-107*, and one down-regulated circRNA may bind to *hsa-miR-151a-5p* (Figure 3B). Based on the above information, we constructed the ceRNA network (mRNA-miRNA-circRNA) (Figure 3C), whose basic pattern of interaction relationship roughly follows that mRNA is up--regulated, miRNA is down-regulated, and circRNA is up-regulated, as opposed to mRNA is down-regulated, miRNA is up--regulated, and circRNA is down-regulated (supplementary table 1), which includes 11 circRNA-miRNA pairs and 7 miRNA-mRNA pairs. To deeply research the functions of the ceRNA network, we further performed single-gene enrichment analysis for six mRNAs and found that *BID* was closely associated mainly with the positive regulation of Fe gamma R-mediated phagocytosis, Legionellosis and Leishmaniasis; *MAP3K1* is mainly associated with positive regulation of Lysosome、Parkinson disease、Protein processing in endoplasmic reticulum, and negative regulation of Autophagy-animal、Coronavirus disease - COVID-19, Fatty acid elongation; *MPO* is mainly associated with positive regulation of Protein processing in endoplasmic reticulum, Ribosome, Th17 cell differentiation, and negative regulation of B cell receptor signaling pathway Chemokine signaling pathway, Cytokine-cytokine receptor interaction; *MYL6* is mainly associated with the positive regulation of Chemical carcinogenesis- reactive oxygen species Diabetic cardiomyopathy, Huntington disease; *PPKCH* was mainly associated with positive regulation of Ribosome. Th1 and Th2 cell differentiation. Th17 cell differentiation and negative regulation of Chemical carcinogenesis –reactive oxygen species. Diabetic cardiomyopathy Non-alcoholic fatty liver disease; *TNFRSF1B* is mainly associated with positive regulation of B cell receptor signaling pathway、Chemokine signaling pathway and Fc gamma R-mediated phagocytosis (Figure 3D).

Immune cell composition in LAML

The stacked, heat, and box plots of Figures 4A, 4B, and 4C show the percentage of 22 immune cells detected by the CIBERSORT algorithm. The relationship between immune cells and prognosis was then analyzed and only Mast cells resting levels were found to be significantly associated with prognosis in LAML.

Co-expression analysis

We performed correlation analysis by Pearson correlation analysis between 22 immune cells and between 22 immune cells and the above prognostically significant 4 miRNAs and 6 mRNAs (Figure 5A, 5B), and found that Monocytes correlated with *BID*, *TNFRSF1B*, *MYL6* expression, Mast cells resting levels were significantly and positively correlated with *MPO* expression, T cells CD4 memory resting with *BID*, *TNFRSF1B*, *MYL6* expression, B cells naïve with *BID*, *TNFRSF1B* expression, Monocytes with *MPO* expression, Mast cells resting levels were significantly negatively correlated with TNFRSF1B expression (Figure 5C).

Immune checkpoint and ceRNA members are expressed in four leukemia types

Immune checkpoints are negative regulators of T cell activation, T cell proliferation and effector functions. ICB is a promising approach to activate anti-tumor immunity [21]. Therefore, we evaluated the correlation between the above mentioned four miRNAs and six mRNAs with significant prognosis and immune checkpoints (Figure 6A). Significant negative correlations were found between *MPO* and *IL2A*, and significant positive correlations were found between *BID* and *TLR4*, *BID* and *ITGB2*, *PRKCH* and *TIGIT*, *hsa-miR-151a-5p* and *SLAMF7*, *TNFRSF1B* and *ITGB2*, *MYL6* and *TLR4*, and *TNFRSF1B* and *TLR4* (Figure 6B). In addition, we analyzed the relationship between mRNA in ceRNA and four leukemia types, and the results showed that all four leukemia types were associated with gene expression profiles, except *MAP3K1* (Figure 6C).

Discussion

LAML is a malignant leukemia caused by the abnormal proliferation and differentiation of stem cells in the bone marrow [1]. LAML has a rapid onset and progresses rapidly, easily leading to serious complications such as anemia, bleeding, and infection, and even if remission is achieved through treatment, LAML patients still have a high rate of relapse [22]. Moreover, there are large prognostic differences between patients and an applicable molecular marker has not been identified to predict the prognosis of patients [23]. Chemotherapy is currently the main treatment, but its efficacy is influenced by several factors [24]. Therefore, further exploration of the disease is needed to validate it. Though the ceRNA network model of LAML has been studied [19], the use of circRNA to identify potential biomarkers of LAML has not yet been reported. We attempted to construct a mRNA-miRNA-circRNA ceRNA network related to LAML immunoinfiltration and prognosis. It may provide clues for future studies on prognostic biomarkers and promising therapeutic targets for LAML.

In our study, 4 miRNAs and 6 mRNAs were screened, and upstream circRNAs were searched based on miRNAs to construct a ceRNA network associated with immune infiltration and prognosis of LAML. Among them, a series of studies reported that *MAP3K1* [25], *MPO* [26], *MYL6* [27], *BID* [28], *PPKCH* [29], *hsa-miR-362-3p* [30], *hsa-miR-151a-5p* [31], and *hsa-miR-19a-3p* [32] could affect the development of LAML. *Hsa-miR-107* [33] and *TNFRSF1B* [34] is associated with the development of chronic granulocytic leukemia. Our study is broadly consistent with the above results, and all of these genes are aberrantly expressed in LAML immune cells to varying degrees. Therefore, we believe that these ceRNA network key factors may be very effective biomarkers for the diagnosis of LAML.

Study has been shown that immune infiltrating cells (TIICs) are key to monitoring the immunogenicity of tumors [35]. TIICs are immune cells present in tumor tissues, including T cells, B cells, natural killer cells, and macrophages, which can play an important anti-tumor role [36]. It has been shown that T-cell infiltration in LAML correlates with patient survival, with higher levels of infiltration associated with higher patient survival [37]. Decreased numbers and activity of NK cells may promote the development of LAML [38]. The decrease in the number and function of DCs may affect the immune response and promote the development of LAML [39]. Therefore, an in-depth study of the role and regulatory mechanisms of TIICs in tumors is important for understanding the mechanisms of tumorigenesis and development and formulating effective tumor immunotherapy strategies. We also found different ratios of many immune cells in LAML tissues. mast cells resting was significantly associated with the prognosis of LAML. Following this, correlations between immune cells and between immune cells and key factors of the ceRNA network were analyzed and some immune cells were found to be significantly associated with ceRNA network key factor expression. Based on the above results, we infer that further understanding of the relationship between ceRNA networks and TIICs can help explore the formation and regulatory mechanisms of the tumor microenvironment, as well as the molecular mechanisms of tumor development and progression, which may provide new ideas and targets for the development of LAML therapeutic strategies targeting the tumor microenvironment.

Immune checkpoint inhibitors are a new type of tumor treatment drugs [40]. They can activate the patient's own immune system by inhibiting immune checkpoint molecules to enhance the immune response of the tumor, thus achieving the effect of treating the tumor [41]. Immune checkpoint inhibitors have also been widely studied and used in the treatment of LAML [42]. Several clinical trials have demonstrated the efficacy and safety of *PD-1/PD-L1* inhibitors and *CTLA-4* inhibitors in the treatment of LAML [43-45]. In our study, ceRNA network key factors were found to be significantly associated with immune checkpoint genes. These results above may offer further elucidation on the mechanism behind immune cell infiltration and adverse prognosis in LAML.

A ceRNA network associated with LAML survive value and immune cell infiltration was constructed by combining mRNA-miRNA and miRNA-circRNA relationships, providing new ideas for our understanding of gene regulation mechanisms. However, its limitations also exist. The ceRNA network model is based on the assumption of competitive endogenous RNAs [46], and this assumption still has many unknown and complex details, such as the selection and identification of miRNA targets [47], the stability and plasticity of ceRNA networks [48], which may affect the precision and reliability of ceRNA networks. We still need a lot of experimental

to verification in the future.

Declarations

Data availability

Our research data sets are available from corresponding authors upon reasonable request. The data set for this study can be found in the database listed in the article. There was no necessity to obtain Ethics Committee approval since all information were publicly available and open-access.

Author Contributions

Hongxia Yao, Zhixia Wei and Xiangjun Fu drafted the manuscript; Xiangjun Fu and Min Yang performed the data analysis; Yueqing Chen performed the sample collection and information recording; Qinxiang Liu performed the manuscript modification; Hongxia Yao, Zhixia Wei and Xiangjun Fu conceived and supervised the study.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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